Supplementary Materials for the manuscript 'Functional characterization of a novel opioid, PZM21, and its influence on behavioural responses to morphine' by Kudla et al.

Synthesis of PZM21

The synthesis of PZM21 was achieved, using commercially available L-tyrosinamide which in two-step sequence was converted into the first semi-product (*S*)-4-[3-amino-2-(dimethylamino)propyl]phenol with specific optical rotation $[\alpha]_D^{25}$ = –9.4°(c 0.47, H_2O). Synthesis of the second semi-product was realized by Henry reaction of thiophene-3-carbaldehyde with nitroethane affording the nitropropene derivative, which was converted into the racemic (*RS*)-1-(thiophen-3-yl)propan-2-amine. For enantiomeric enrichment, chiral resolution of the racemic primary amine via repetitive crystallization with di-*p*-anisoyl-(*S*)-tartaric acid was performed. Triple crystallization delivers the product with $[\alpha]_D^{25}$ = +17.5°(c 0.92, H_2O). Before the final coupling of both semi-products, the (*S*)-1-(thiophen-3-yl)propan-2-amine with 4-nitrophenyl chloroformate was activated. Specific optical rotation for obtained 4-nitrophenyl (*S*)-[1-(thiophen-3-yl)propan-2-yl]carbamate: $[\alpha]_D^{25}$ = –43.9°(c 0.49, $CHCl_3$). All chemicals and solvents were purchased from Sigma Aldrich, Merck or Combi-Blocks and were used without additional purification. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance III HD 400 NMR spectrometer. Sample was dissolved in chloroform-d with TMS as the internal standard. The spectral data of the compound refer to its free base. ^1H NMR (400 MHz) δ 7.23 (dd, J = 4.9, 2.9 Hz, 1H), 7.01- 6.91 (m, 4H), 6.78 (d, J = 8.4 Hz, 2H), 5.00–4.80 (m, 1H), 4.90 (br. s, 1H), 3.97 (hept, J = 6.7 Hz, 1H), 3.36–3.25 (m, 1H), 3.00–2.66 (m, 6H), 2.35 (s, 6H), 2.27 (dd, J = 13.5, 10.6 Hz, 1H), 1.09 (d, J = 6.6 Hz, 3H). 13C NMR (101 MHz) δ 158.3, 155.6, 138.5, 129.9 (2C), 129.2, 129.0, 125.3, 122.0, 115.8 (2C), 65.9, 46.7, 40.3, 40.1 (2C), 37.5, 30.5, 20.7. Free base was converted into hydrochloride salt. Elemental analysis was carried out with a Perkin Elmer 2400 ($C_{19}H_{27}N_3O_2S \cdot HCl \cdot 1.4H_2O$): found C: 54.20%, H: 7.30%, N: 9.56%; requires C: 53.90%, H: 7.33%, N: 9.93%.

Chiral HPLC: AS-H column, 0.1% diethyl amine in isopropanol/hexane 35:65, 0.8 ml·min^{-1}, 20 min: t_R = 13.8 min. The stereoisomeric purity was determined to be > 99%.

Analytical chiral HPLC was performed on HPLC Smartline, Knauer combined with UV detector 2600 (detection λ = 254 nm) using Chiralpak® AS-H column at 21 °C, 0.8 ml·min^{-1} flow rate and 20 μL injection volume (the sample was dissolved in LC-MS grade isopropanol). UPLC/MS analysis was performed on Waters TQD spectrometer combined with UPLC Acquity H-Class with PDA eLambda detector. Waters Acquity UPLC BEH C18 1.7 μm 2.1 × 100 mm chromatographic column was used, at 40 °C, 0.300 ml·min^{-1} flow rate and 1.0 μL injection volume (the sample was dissolved in LC-MS grade acetonitrile). Mass spectrum was recorded under electrospray ionization in positive mode (ESI+) and chromatogram was recorded with UV detection in the range of 190–300 nm. The gradient conditions used were: 80% phase A (water + 0.1% formic acid) and 20% phase B (acetonitrile + 0.1% formic acid) to 100% phase B (acetonitrile + 0.1% formic acid) at 3.0 minutes, kept till 3.5 minutes, then to initial conditions until 4.0 minutes and kept for additional 2.0 minutes. Total time of analysis – 6.0 minutes. LCMS (ESI): t_R = 1.6 min, purity: 97%; m/z found: 362.1, calcd.: 362.2 ($[M+H]^+$).

HPLC data

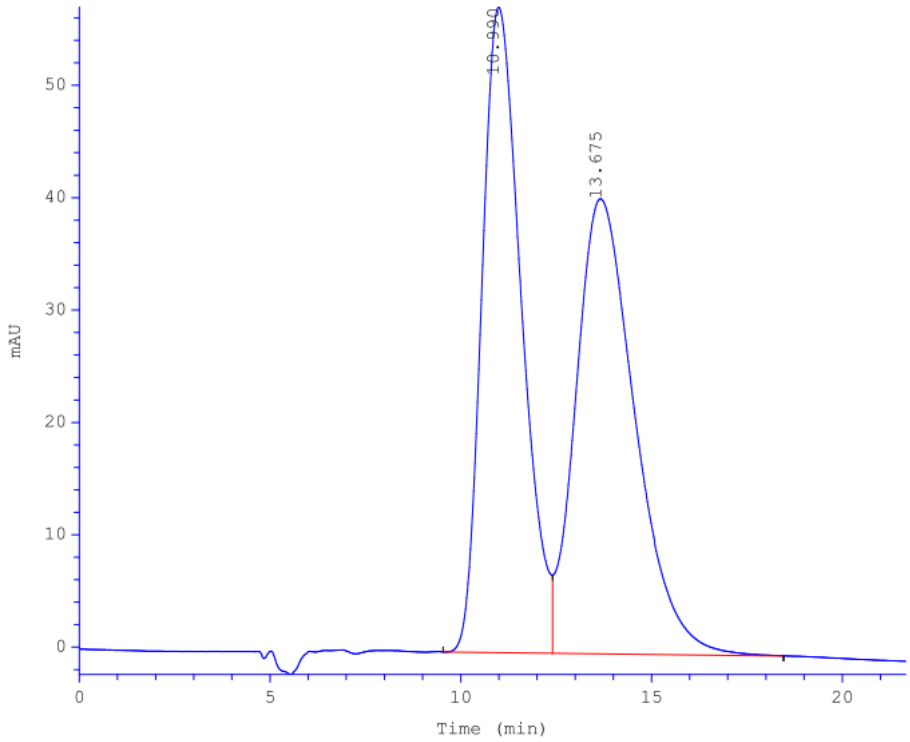

Fig. S1 HPLC data of 1-[(*S*)-2-(Dimethylamino)-3-(4-hydroxyphenyl)propyl]-3-((*R/S*)-1-(thiophen-3-yl)propan-2-yl)urea. HPLC (Chiralpak® AS-H; 0.1% diethyl amine in isopropanol/hexane 35:65, 0.8 ml·min^{-1} T = 21 °C); t_R = 11.0 min (*S*,*R*); t_R = 13.7 min (*S*,*S*). Specific values are indicated in **Table S1.**

Table S1. HPLC data of 1-[(*S*)-2-(Dimethylamino)-3-(4-hydroxyphenyl)propyl]-3-((*R/S*)-1-(thiophen-3-yl)propan-2-yl)urea.

	Ret.time [min]	*Start [min]*	*End [min]*	*Area [mAU*min]*	*Height [mAU]*	*% Area*	*Width [min]*
1	*10.990*	*9.54*	*12.41*	*69.1195*	*57.4352*	*48.6647*	*1.063*
2	*13.675*	*12.41*	*18.46*	*72.9126*	*40.5031*	*51.3353*	*1.573*

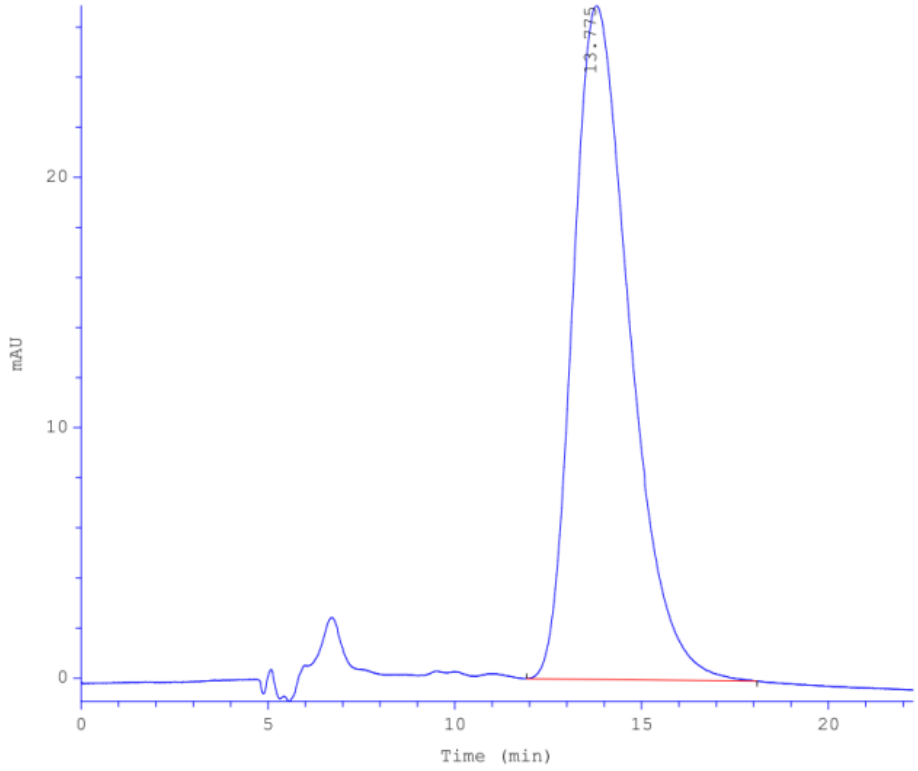

Fig. S2 1-[(S)-2-(Dimethylamino)-3-(4-hydroxyphenyl)propyl]-3-((S)-1-(thiophen-3-yl)propan-2-yl)urea – PZM21. HPLC (Chiralpak® AS-H; 0.1% diethyl amine in isopropanol/hexane 35:65, 0.8 ml·min^{-1} T = 21 °C); tR = 13.8 min (S,S). Specific values are indicated in **Table S2.**

Table S2. HPLC data of 1-[(*S*)-2-(Dimethylamino)-3-(4-hydroxyphenyl)propyl]-3-((*S*)-1-(thiophen-3-yl)propan-2-yl)urea – PZM21

	Ret.time [min]	*Start [min]*	*End [min]*	*Area [mAU*min]*	*Height [mAU]*	*% Area*	*Width [min]*
1	*13.775*	*11.92*	*18.09*	*47.5786*	*26.9075*	*100.0000*	*1.626*

Radioligand competition binding and [^{35}S]GTPγS binding assay

Animals. For membrane homogenate preparations, male Wistar rats (250-300 g body weight, RRID:RGD_13508588) were used, which were previously housed in the local animal house of BRC (Szeged, Hungary). Animals were kept in a temperature-controlled room (21-24°C) under a 12/12 h light and dark cycle and were provided with water and food ad libitum. All housing and experiments were performed in accordance with the European Communities Council Directives (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§). The total number of animals as well as their suffering was minimized.

Chemicals. Tris-HCl, EGTA, NaCl, $MgCl_2$ x $6H_2O$ was purchased from Sigma-Aldrich (Hungary). The highly selective μ-OR agonist enkephalin analog Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) was obtained from Bachem Holding AG (Switzerland), the highly selective δ-OR agonist Ile^{5,6}-deltorphin II (IleDelt II) was synthesized in the Laboratory of Chemical Biology group of the Biological Research Center (BRC, Hungary). All ligands were dissolved in water and were stored as a 1 mM stock solution at -20˚C. [^{3}H]DAMGO (specific activity: 38.8 Ci·mmol^{-1}) and [^{3}H]IleDelt II (specific activity: 19,6 Ci·mmol^{-1}) (Ioja *et al*., 2007) were radiolabeled by the Laboratory of Chemical Biology group in the BRC (Hungary). The radiolabeled GTP analog, [^{35}S]GTPγS (specific activity: 1000 Ci·mmol^{-1}) was purchased from Hartmann Analytic (through Izotóp Intézet Kft., Hungary). An UltimaGold^{TM} MV aqueous scintillation cocktail was purchased from PerkinElmer (through Per-Form Hungária Kft., Hungary).

Rat membrane preparations. Rats were decapitated, and their brains were quickly removed. The brains were prepared for membrane preparation according to Benyhe *et al*. (1997) for competitive and [^{35}S]GTPγS binding assays. Briefly, the brains were homogenized, centrifuged in ice-cold 50 mM Tris-HCl (pH 7.4) buffer and incubated at 37°C for 30 min in a shaking water bath. After incubation, centrifugation was repeated as described previously, and the final pellet was suspended in 50 mM Tris-HCl pH 7.4 buffer containing 0.32 M sucrose and stored at -80°C. For the **[**^{35}S]GTPγS binding assay, the final pellet was suspended in ice-cold TEM (Tris-HCl, EGTA, $MgCl_2$) buffer and stored at –80°C for further use.

Competition radioligand binding. Compounds were incubated together with rat brain membranes containing 0.3-0.5 mg·ml^{-1}of protein in increasing concentrations (0.1 nM – 10 μM) together with ~1 nM [^{3}H]DAMGO or [^{3}H]IleDelt II at 35˚C for 45 min. Total and nonspecific binding was determined in the absence of ligands and presence of 10 μM unlabeled naloxone, respectively. The bound and unbound radioligands were separated by rapid vacuum filtration through Whatman GF/C glass fibers and washed three times with 5 ml of ice-cold 50 mM Tris-HCl (pH: 7.4). The radioactivity of the filters was detected in UltimaGold^{TM} MV aqueous scintillation cocktail with a Packard Tricarb 2300TR liquid scintillation counter. The competitive binding assays were performed in duplicate and repeated at least three times.

[^{35}S]GTPγS binding assay. The functional [^{35}S]GTPγS binding experiments were performed as previously described (Traynor and Nahorski, 1995), with slight modifications. Briefly, the rat brain membrane homogenates containing ~ protein were incubated at 30˚C for 60 min in Tris-EGTA buffer (pH 7.4) composed of 50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, and 100 mM NaCl. The incubation mixture further contained 0.05 nM [^{35}S]GTPγS, increasing concentrations (0.1 nM-10 μM) of PZM21 and DAMGO and excess GDP (30 μM). The final

volume of the incubation mixture was 1 ml. Total binding was measured in the absence of the test compounds, while nonspecific binding was determined in the presence of 10 μM unlabeled GTPγS. The bound and unbound [^{35}S]GTPγS was separated by rapid filtration under vacuum (Brandel M24R Cell Harvester) and washed three times with 5 ml of ice-cold 50 mM Tris-HCl through Whatman GF/B glass fibers (GE Healthcare Life Sciences through Izinta Kft., Hungary). The radioactivity of the filters was detected as described in the radioligand binding experiments section. [^{35}S]GTPγS binding experiments were performed in triplicate and repeated at least three times.

Data analysis. The specific binding of the radiolabeled compounds was calculated by subtracting nonspecific binding levels from total binding and was given as a percentage. The data were normalized to total specific binding, which was established as 100% (in [^{35}S]GTPγS assays, it also represents basal activity). Experimental data were presented as the means ± SEM. Data obtained from competitive binding experiments were fitted with a professional curve fitting program, GraphPad Prism 7.0 (GraphPad Prism Software Inc., USA, RRID:SCR_002798), using nonlinear regression, the 'One-site competition' equation to determine the logIC_{50} value (unlabeled ligand affinity). The K_i value was calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). In [^{35}S]GTPγS assays, data were fitted using nonlinear regression, applying the 'Sigmoid dose-response' equation to determine the maximum G-protein efficacy (E_{max}) and ligand potency (EC_{50}). Statistical analyses were performed with GraphPad Prism 5.0 applying unpaired Student's t-test with a two-tailed p value. Data were considered significant at the $p<0.05$ level.

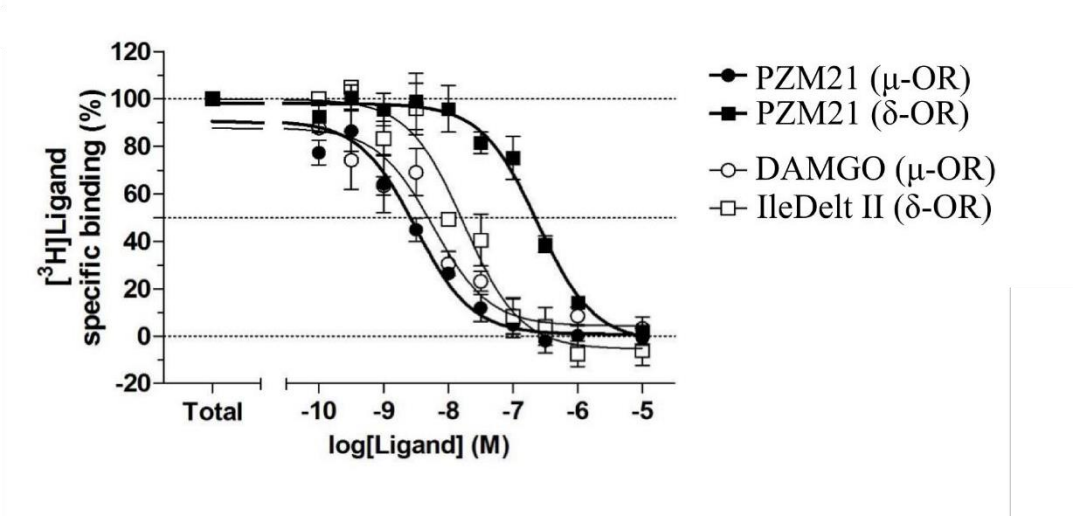

Fig. S3 The μ-OR and δ-OR competitive binding of PZM21 in rat brain membrane homogenates. Data represent the specific binding of the radioligands to μ-ORs and δ-ORs in the presence of [^{3}H]DAMGO, [^{3}H]IleDelt II or PZM21. 'Total' on the x-axis indicates the total specific binding of the radioligands in the absence of the compounds. The figure also indicates the level of 50% radioligand specific and nonspecific binding (0%), highlighted with dotted lines. Points represent means ± SEM for at least three experiments performed in duplicates. The affinity values are indicated in **Table S3**.

Table S3 The μ-OR and δ-OR affinity values of PZM21.

	logIC_{50} ± S.E.M. (M) (K_i[1], nM)	
	μ-OR ([^{3}H]DAMGO)	**δ-OR ([^{3}H]IleDelt II)**
PZM21	-8.50 ± 0.10 (3.14)	-6.61 ± 0.10* (241.2)
DAMGO	-8.23 ± 0.13 (5.89)	-
IleDelt II	-	-7.75 ± 0.11 (17.6)

[1]: calculated by the Cheng-Prusoff equation based on the logIC_{50} value of the compound and the given radioligand concentration and dissociation constant (K_d)

*: significant reduction in logIC_{50} value compared to μ-OR affinity of PZM21 (***$p<0.05$)

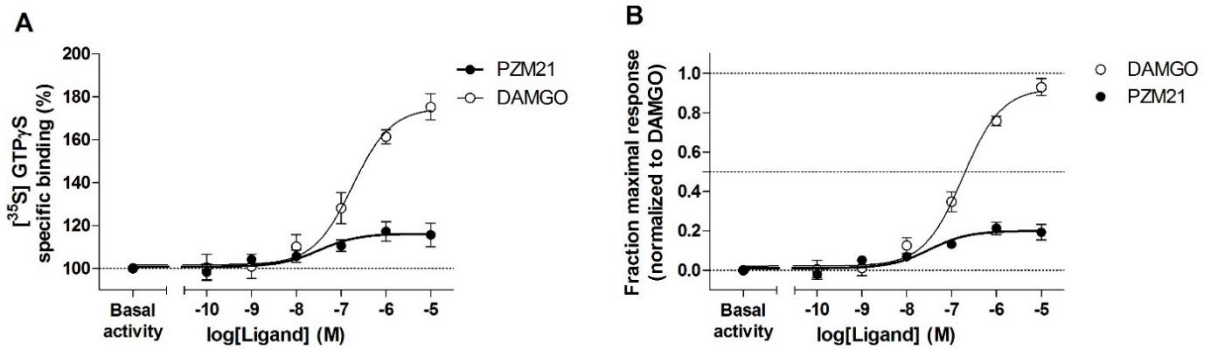

Fig. S4 The G protein activity of PZM21 compared to DAMGO in [^{35}S]GTPγS binding assays performed in rat brain membrane homogenates. **[A]** Data represents the specific binding of [^{35}S]GTPγS binding in percentage over basal activity (100%) in the presence of increasing concentrations of PZM21 or DAMGO for control. **[B]** Data represents the fractional maximal response (settled as 1) of PZM21 normalized to DAMGO in the presence of increasing concentrations of PZM21 or DAMGO. The figure indicates the level of half maximal response (0.5) with a dotted line. In both figures 'basal activity' on the x-axis indicates the total specific binding of the [^{35}S]GTPγS in the absence of the compounds, which was settled as 100 % or 0 (indicated with a dotted line) and also represents the monitored G protein basal activity. Points represent means ± SEM for at least three experiments were performed in duplicate. The curve parameters are indicated in **Table S4**.

Table S4 The G protein activity of PZM21 compared to that of DAMGO.

	logEC_{50} ± S.E.M. (M) (EC_{50}, nM)	**E_{max} ± S.E.M.**	
		(%)	**(fraction normalized to DAMGO)**
PZM21	-7.49 ± 0.29 (32.4) (-8.20 ± 0.09)[1]	116.2 ± 1.5	0.19 ± 0.02 (1.02 ± 0.03)[1]
DAMGO	-6.74 ± 0.09 (179.1) (-8.13 ± 0.08)[1]	174.8 ± 2.8	0.92 ± 0.03 (0.93 ± 0.04)[1]

[1]: Reported by Manglik *et al.* (2016) in HEK 293 cells coexpressing the human μ-OR and the PTX-insensitive G protein subunit $G_{\alpha i2}$

Supplementary data on brain microdialysis experiments

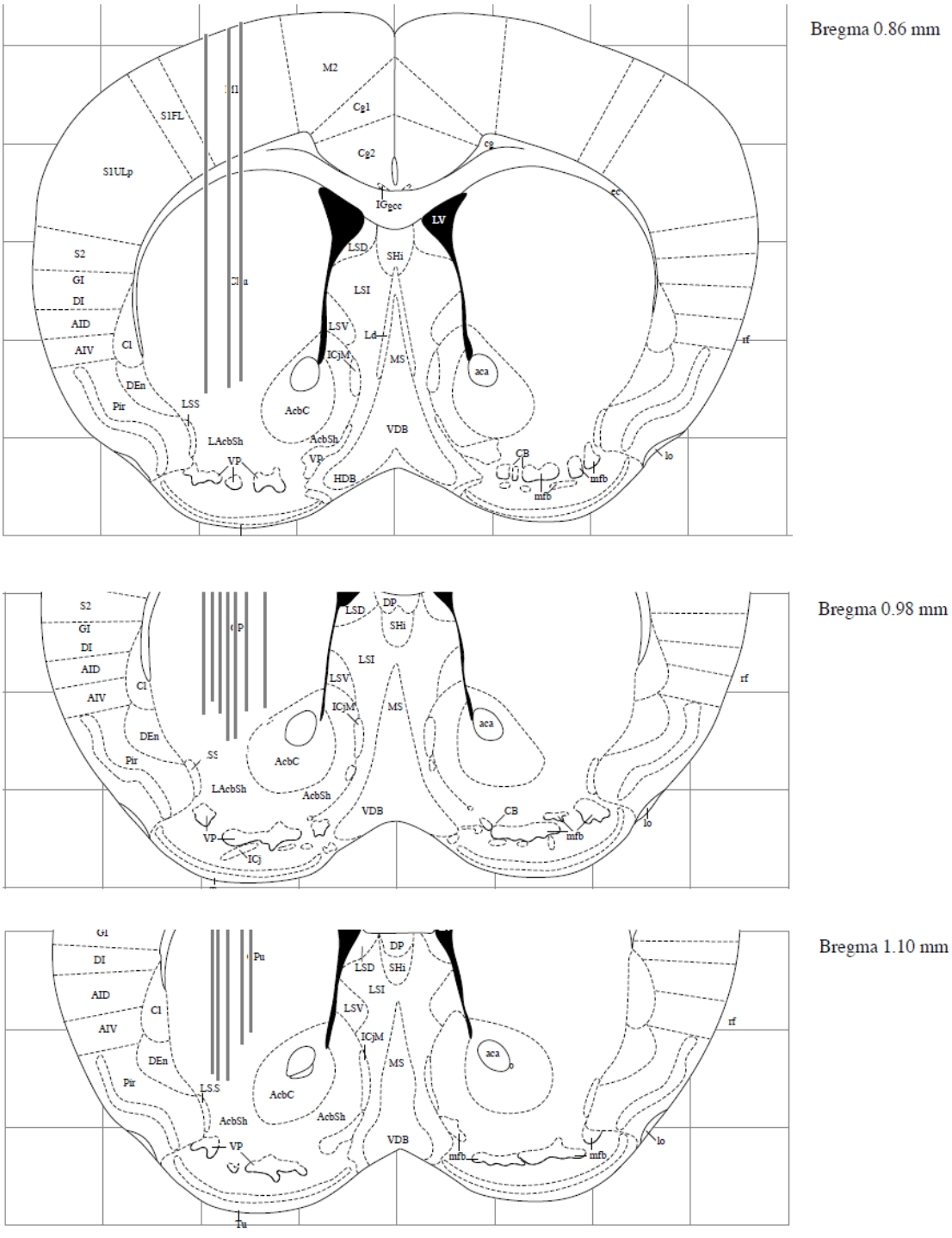

Fig. S5 Presentation of the microdialysis probe placement in the mouse striatum.

Supplementary data on behavioural experiments

Table S5 Numbers of animals used in behavioural experiments.

Figure	Treatment group	Number of animals
1A	Sal	n=9
	Morph 10	n=7
1B	Sal, PZM21 80	n=9
	PZM21 20, PZM21 40	n=8
1C	Sal+PZM21 40	n=11
	Cyp 10+PZM21 40	n=9
1D	Sal, PZM21 20, PZM21 40, PZM21 80	n=10
	Morph 10	n=7
1E	Sal+PZM21 40	n=12
	Cyp 10+PZM21 40	n=9
1F	Sal, PZM21 5	n=6
	PZM21 2,5, PZM21 7,5	n=7
2A	Sal, PZM21 20, PZM21 40, PZM21 80, Morph 10	n=10
2B	Sal, PZM21 40, Morph 10	n=9
	PZM21 20, PZM21 80	n=10
2C	Sal, PZM21 20, PZM21 40, PZM21 80	n=10
	Morph 10	n=16
2D	Sal, PZM21 20, PZM21 40	n=9
	PZM21 80	n=10
3A-C	Sal	n=6
	PZM21 0,05, PZM21 0,5	n=8
	Oxy 0,06	n=13
3D	Sal	n=6
	PZM21 0,05, PZM21 0,5	n=8
	Oxy 0,06	n=11
4A-B	Sal, PZM21 40, PZM21 80, Morph 10, Morph 20	n=6
5A	Sal+Morph 5, PZM21 20+Morph 5, PZM21 40+Morph 5	n=10
5B	Sal+Morph10, PZM21 20+Morph 10	n=8
	PZM21 40+Morph 10	n=10
5C	Sal+Morph10	n=14
	PZM21 20+Morph 10	n=10
	PZM21 40+Morph 10	n=13
5D	Sal+Morph10	n=12
	PZM21 20+Morph 10	n=10
	PZM21 40+Morph 10	n=9

REFERENCES:

Benyhe, S., Farkas, J., Tóth, G., and Wollemann, M. (1997). Met5-enkephalin-Arg6-Phe7, an endogenous neuropeptide, binds to multiple opioid and nonopioid sites in rat brain. J. Neurosci. Res. *48*: 249–258.

Cheng, Y., and Prusoff, W.H. (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochemical Pharmacology *22*: 3099–108.

Ioja, E., Tourwé, D., Kertész, I., Tóth, G., Borsodi, A., and Benyhe, S. (2007). Novel diastereomeric opioid tetrapeptides exhibit differing pharmacological activity profiles. Brain Research Bulletin *74*: 119–29.

Manglik, A., Lin, H., Aryal, D.K., McCorvy, J.D., Dengler, D., Corder, G., et al. (2016). Structure-based discovery of opioid analgesics with reduced side effects. Nature *537*: 185–190.

Traynor, J., and Nahorski, S. (1995). Modulation by mu-opioid agonists of guanosine-5'-O-(3-[35S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. Molecular Pharmacology *47*: 848–54.